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Induction of Competing Apoptotic and Survival Signaling Pathways in the Macrophage by the Ribotoxic Trichothecene Deoxynivalenol

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Deoxynivalenol (DON) and other ribotoxic trichothecenes cause immune stimulation and suppression in leukocytes by upregulating gene expression and apoptosis, respectively. The purpose of this study was to test the hypothesis that MAPKs mediate both apoptosis and survival in DON-exposed macrophages. At concentrations which partially inhibit translation, DON induced phosphorylation of p38 and ERK 1/2 mitogen activated protein kinases within 15 min in RAW 264.7 macrophages and these effects lasted up to 3 h. DON-exposed cells exhibited marked caspase 3-dependent DNA fragmentation after 6 h which was suppressed and attenuated by the p38 inhibitor SB203580 and ERK inhibitor PD98059, respectively. DON readily induced the phosphorylation and activity of p53 and this was inhibitable by SB203580. DON exposure evoked BAX translocation to mitochondria and corresponding cytochrome C release but did not alter mitochondrial membrane potential. The p53 inhibitor PFTα reduced both DON-induced phosphorylation of p53 and p53 binding activity. Moreover, both PFTα and p53 siRNA transfection suppressed DON-induced caspase-3 activity and subsequent DNA fragmentation. Concurrent with p53 activation, DON activated two anti-apoptotic survival pathways as evidenced by both ERK-dependent p90 Rsk and AKT activation. Taken together, the results indicate that DON initiates competing apoptotic (p38/p53/Bax/Mitochondria/Caspase-3) and survival (ERK/AKT/p90Rsk/Bad) pathways in the macrophage.

Key Words: **deoxynivalenol; trichothecene; apoptosis; macrophages; ribotoxic.**

Trichothecene mycotoxins are a diverse group of sesquiterpenoid metabolites (Grove, 1988, 1993, 2000) that are produced by fungi encountered in food and the environment (Pestka *et al.*, 2004). The most frequently encountered trichothecene, deoxynivalenol (DON or ''vomitoxin''), is produced by *Fusarium* and commonly enters cereal-based foods, making it a human health concern (Pestka and Smolinski, 2005). Trichothecenes have also been linked to indoor air illness (Ammann, 2003; Sudakin, 2003) and chemical warfare (Etzel, 2002; Zapor and Fishbain, 2004) as well as have been evaluated for cancer chemotherapy (Adler *et al.*, 1984; Bukowski *et al.*, 1982). A critical target of DON and other trichothecenes is the immune system. Numerous studies conducted on host resistance, antibody responses, and cell mediated immunity have revealed that trichothecenes stimulate or suppress immune function depending on dose, exposure frequency, and timing of functional immune assay (Pestka *et al.*, 2004). As observed *in vivo,* these toxins are stimulatory in some leukocyte models but inhibitory in others; paradoxically, these activities sometimes co-occur.

The macrophage and innate immune system appear to be exquisitely sensitive to trichothecenes. Stimulation of macrophages by low doses or concentrations of trichothecenes upregulate expression of inflammation-related genes *in vivo* and *in vitro* including COX-2 (Moon and Pestka, 2003a,b), proflammatory cytokines (Wong *et al.*, 1998; Zhou *et al.*, 1997), nitric oxide synthase (Ji *et al.*, 1998), and numerous chemokines (Chung *et al.*, 2003a; Kinser *et al.*, 2004). In contrast, exposure to high doses or concentrations of trichothecene can induce apoptosis in macrophages (Yang *et al.*, 2000; Zhou *et al.*, 2003a) thereby suppressing innate immune function (Pestka *et al.*, 2004).

The underlying molecular mechanisms for the paradoxical effects of trichothecenes on leukocytes and, ultimately, the overall immune system are not completely resolved. The most prominent molecular target of trichothecenes is the 60S ribosomal subunit suggesting that one underlying mechanism is translational inhibition (Ueno, 1984). However, it is now known that trichothecenes and other translational inhibitors which bind to ribosomes can also rapidly activate mitogen-activated protein kinases (MAPKs) and induce apoptosis in a process known as the ''ribotoxic stress response'' (Iordanov *et al.*, 1997; Laskin *et al.*, 2002). MAPKs modulate physiological processes including cell growth, differentiation, and apoptosis (Cobb, 1999) and are critical for signal transduction in the immune response (Dong *et al.*, 2002). MAPK subfamilies include (1) p44 and p42 MAPKs, also known as

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extracellular signal regulated protein kinase 1 and 2 (ERK1 and 2); (2) p54 and p46 c-Jun N-terminal kinase 1 and 2 (JNK 1/2), and (3) p38 MAPK (Cobb, 1999; Schaeffer and Weber, 1999; Widmann *et al.*, 1999).

The demonstration that trichothecenes activate JNK, ERK, and p38 *in vitro* (Moon and Pestka, 2002; Shifrin and Anderson, 1999; Yang *et al.*, 2000; Zhou *et al.*, 2003a) and *in vivo* (Zhou *et al.*, 2003b) suggests that the ribotoxic stress response might mediate trichothecene immunotoxicity. DON induces ERK 1/2, p38, and JNK 1/2 phosphorylation in the RAW 264.7 macrophage cell line. ERK and p38 but not JNK are involved in trichothecene-induced transactivation of TNF-α and COX-2 in this cell line, whereas p38 appears to be involved in trichothecene-mediated mRNA stability (Chung *et al.*, 2003b, Moon and Pestka, 2002, 2003a,b; Moon *et al.*, 2003). Yang *et al.* (2000) found that trichothecene-mediated cytotoxicity and apoptosis correlate closely with activation of all three different MAPK in RAW 264.7 macrophage cells and U937 monocyte models suggesting possible involvement of these kinases in trichothecene-induced apoptosis.

Apoptosis in the immune system is orchestrated by two general categories of proteases known as caspases (Green, 2003). The executioner caspases including caspase-3 exist as inactive dimers which are activated by initiator caspases. Two distinct pathways for apoptosis (Downward, 2004) exist based on the types of initiator caspases and adapter molecules that bind them. The intrinsic pathway is initiated by mitochondria, which are regulated by Bcl-2 protein family. The anti-apoptotic Bcl-2 and Bcl-xL reside in the outer mitochondrion membrane and inhibit cytochrome c release. In response to death stimuli, the pro-apoptotic Bad and Bax translocate to the mitochondrial membrane and form a proapoptotic complex with Bcl-xL or Bcl-2. This complex induces pores in the mitochondrial membrane leading to cytochrome C release. Caspase-9 is activated by cytochrome C, which complexes with Apaf-1 initiating activation of caspase-3 which is an "executioner" that degrades a variety of cellular components producing apoptosis. In contrast to the intrinsic pathway, the extrinsic apoptotic pathway is initiated by death receptors such as (e.g., FAS, TNFR) and their respective ligands. Death receptor ligands initiate signaling via receptor oligomerization, which in turn result in the recruitment of specialized adaptor proteins and activation of caspase cascades.

The purpose of this study was to test the hypothesis that MAPKs mediate both apoptosis and survival in DON-exposed macrophages. RAW 264.7 murine macrophage cells were used as the model for apoptosis based on its previously described susceptibility to trichothecenes (Yang *et al.*, 2000). The results indicate that DON induced the intrinsic pathway of apoptosis via phosphorylation of p38 and p53. DON concurrently induced survival pathways mediated by phosphorylation of ERK and p90Rsk as well as AKT, both of which drive multiple anti-apoptotic pathways.

MATERIALS AND METHODS

Cell culture. RAW 264.7 murine macrophage cells (American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma Chemical Co., St. Louis, MO) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (HIFBS, Sigma), 100 units/ml penicillin, and 100 μg/ml streptomycin (Sigma) in a 5% CO_2 humidified incubator at 37°C. Macrophage cell number and viability were assessed by tryphan blue (Sigma) dye exclusion using a hematocytometer and were verified to exceed 95% in each study. Cells (5×10^5/ml) were seeded in 10 ml of medium in a 100 mm^2 sterile tissue culture dishes overnight to achieve 80% confluency. DON concentrations which partially inhibit translation (250–500 ng/ml) were used for assessing competing pathways. These concentrations are known to induce both gene induction and apoptosis in RAW 264.7 cells (Zhou *et al.*, 2003a). Concentrations of MEK1/ERK 1/2 inhibitor PD98059 (30 μM) and p38 inhibitor SB203580 (5 μM) based on previous studies employing these inhibitors and their inability to induce significant cytotoxicity in RAW 264.7 cells as determined by MTT assay.

Western analysis. Cells were washed with ice-cold phosphate-buffered saline (PBS), lysed in boiling lysis buffer (1%[w/v] Sodium dodecylsulfate, 1 mM sodium ortho-vanadate and 10 mM Tris pH 7.4), boiled for 5 min and sonicated briefly, The lysate was centrifuged at 12,000 × *g* for 15 min at 4°C. Protein was measured in the resultant supernatant with a Bio-Rad DC protein assay kit (Bio Rad Laboratories Inc., Melville, NY). Total cellular proteins were resolved by 8% (w/v) acrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham, Arlington Heights, IL). After blocking with 3% (w/v) bovine serum albumin 20 mM Tris (pH 7.4) containing 1500 mM 0.05% NaCl and Tween 20 immobilized proteins were incubated with phosphospecific antibodies followed by horseradish peroxidase-conjugated anti-rabbit IgG antibodies (Amersham). Bound peroxidase was determined using an ECL Chemiluminescence Detection Kit (Amersham). Western analysis was conducted using primary antibodies specific for phospho-JNK1/2, phospho-ERK1/2, phopho-p38, phospho-p53 (Ser 15), phospho-p21 (Ser 146), phospho-p90Rsk (Ser 380), phospho-Bad (Ser 136), phospho-AKT (Ser 473), phospho-GSK-3β (Ser 9), and phospho-FKRH (Ser 256) (Cell Signaling, Beverly, MA). To assess loading, membranes were stripped and reprobed with specific antibodies that recognize both phosphorylated and unphosphorylated forms (Cell Signaling) of each protein or antibody against housekeeping β-actin.

For determination of the translocation of Bax and cytochrome C, the mitochondrial fraction was separated from cytosolic fraction of cells using an ApoAlert Cell Fractionation Kit (Clontech, Palo Alto, CA). Briefly, cells were suspended in fractionation buffer mix and homogenized with Dounce tissue homogenizer. Cell debris was removed by centrifugation at 700 × *g* for 10 min at 4°C. The supernatant was recentrifuged at 10,000 × *g* for 25 min at 4°C. Resultant supernatant fraction (cytosol) and pellet (mitochondria) were subjected to Western analysis as described above using primary antibodies to Bax (Cell Signaling) and cytochrome C (BD-PharMingen, San Diego, CA).

DNA fragmentation analysis. DNA fragmentation was determined by a modification of the method of Sellins and Cohen (1987). Briefly, cells (1×10^7) in PBS were centrifuged for 5 min (500 × *g*) at 4°C and the pellet suspended in 0.1 ml hypotonic lysing buffer (10 mM Tris, 10 mM EDTA, 0.5% Triton X-100, pH 8.0). Cells were incubated at 4°C for 10 min. The resultant lysate was centrifuged for 30 min (13,000 × *g*) at 4°C. The supernatant containing fragmented DNA was digested for 1 h at 37°C with RNase A (0.4 mg/ml) and then incubated for another hour at 37°C with proteinase (0.4 mg/ml). DNA was precipitated in 50% (v/v) isopropanol and 0.5 M NaCl overnight at −20°C. The precipitate was centrifuged at 13,000 × *g* for 30 min at 4°C. The resultant pellet was air dried, resuspended in 10 mM Tris, 1 mM EDTA, pH 8.0, then electrophoresed at 8.5 V/cm for 2 h in 2% (w/v) agarose gel in 90 mM Tris-borate buffer (pH 8.0) containing 2 mM EDTA. After electrophoresis, gels were stained with ethidium bromide and the nucleic acid bands visualized with a UV transilluminator and photographed.

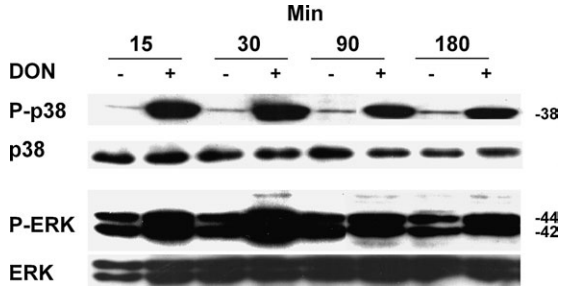

FIG. 1. DON-induces phosphorylation of p38 and ERK 1/2 MAPKs. RAW 264.7 cells (5×10^5/ml) were cultured with 250 ng/ml of DON for various time intervals. Cell lysates (40 μg) were resolved on SDS-PAGE and subjected to Western blot analysis with antibody specific for phosphorylated p38 and ERK. Blots were stripped and reprobed with specific antibodies that recognized both phosphorylated and unphosphorylated forms of each MAPK for assessment of changes in protein loading. Results are representative of three separate experiments.

Caspase-3 assay. Cells were suspended 200 μl of CHAPS buffer (100 mM HEPES [pH 7.5]) containing 10% (v/v) sucrose, 0.5% (w/v) CHAPS, 1 mM EDTA, 10 mM DTT, and protease inhibitor cocktail 2 μl [Sigma], placed on ice for 30 min sonicated briefly, and then centrifuged 10,000 × *g* for 10 min. Following protein assay, lysates (50 μg in 100 ul CHAPS buffer) were incubated at 37°C for 30 min with an equal volume of fluorogenic substrate consisting of 25 μM DEVD-AMC (Calbiochem, San Diego, CA) dissolved in CHAPS buffer. Substrate cleavage was measured using a Cyto Fluor II microplate fluorescence reader (Biosearch, Bedford, MA) at excitation 360 nm and emission wavelengths of 460 nm, respectively.

Electrophoretic mobility shift assay (EMSA). EMSA was used to characterize p53 binding activity in nuclear extracts (Laniel *et al.*, 2001). Briefly, double-stranded p53 consensus probe (Santa Cruz Biotech, Santa Cruz, CA) was radiolabelled with [γ-^{32}P] ATP using Ready to Go Polynucleotide Kinase Kit (Pharmacia Biotech, Piscataway, NJ). Nuclear extracts containing 5 to 10 μg protein were added to DNA-binding reaction buffer consisting of 20 mM HEPES, (pH 7.9) containing 60 mM KCl, 1 mM EDTA, 0.5 mM DTT, and 2 μg poly (dI-dC) in a total volume of 20 μl. These were preincubated on ice for 15 min. to block the nonspecific binding. Following the addition of 1 μl ^{32}P-labeled probe containing 30,000 cpm, the incubation was continued for 30 min at room temperature to promote the formation of nucleoprotein complex. Resultant nucleoprotein complexes were resolved on 4% (w/v) native polyacrylamide gels in 0.5× TBE buffer, which were then dried and visualized by autoradiography.

Immunofluorescence staining. Phospho-p53 was determined by immunofluorescence. RAW cells (5×10^5/ml) were grown on slides and fixed with 4% (w/v) paraformaldehyde for 20 min at 25°C. After washing with PBS for 5 min three times, slides were blocked with 1.5% (v/v) goat serum in PBS for 20 min at 25°C. These were then incubated with anti-phospho-p53 (Ser 15) (Santa Cruz SC-11764 R) for 1 h at 25°C. After washing three more times with PBS, slides were incubated with goat anti-rabbit IgG-FITC conjugates (2 μg/ml) for 30 min at 25°C, washed three times with PBS. Gel/mount (Bio media Corp., Foster City, CA) was added to slide and then sealed with coverslip. Slides were examined under a Nikon Labophot fluorescence microscope (Mager Scientific, Inc., Dexter, MI).

Analysis of mitochondrial membrane potential. Mitochondrial membrane potential ($\Delta\psi$) was analyzed with a MitoProbe JC-1 Assay Kit (Molecular Probes, Eugene, OR), according to manufacturer's protocols. Briefly, RAW 264.7 cells (1×10^6/ml) were incubated in 12 well tissue culture plates with vehicle, DON at various concentrations or 50 μM CCCP (positive control). After appropriate time intervals supernatant was removed. Cells were released with 1 ml of Accutase (Innovative Cell Technologies, San Diego, CA). After centrifugation, cells were suspended in 1 ml PBS containing 2 μM JC-1 dye which exists as a green monomer but accumulates as a red aggregate in mitochondria. Normal mitochondria will appear red following aggregation of the JC-1 reagent which emits at 590 nm (red). Following mitochondrial depolarization, the dye remains in its monomeric form and emits at 529 nm (green). Cell suspensions were incubated in 5% CO_2 37°C for 30 min and supernatant removed by centrifugation from 400 × *g* for 5 min and analyzed by flow cytometry on a Becton Dickinson FACS Vantage (San Jose, CA) using 488 nm excitation with 530 and 585 nm band p457 emission filters (Cossarizza *et al.*, 1993).

siRNA preparation and transfection. p53 siRNAs were generated with a Silencer siRNA Cocktail Kit (Rnase III) (Ambion Inc., Austin, Texas). Briefly, total RNA was extracted from RAW 264.7 cells with TRIZOL (Invitrogen, Carlsbad, CA) and reverse transcribed with a RETRO Script Kit (Ambion) to produce cDNA, PCR primers containing T7 RNA polymerase promoters were designed to amplify a 217 bp fragment of murine macrophage p53 gene (641 bp from the 5′ end), and had the following sequences: forward 5′TAATACGACCACTATAGGGCACAGCGTGGTGGTACCTTA and reverse 5′-TAATACGACTCACTATAGGGCTTCTGTACGGCGGTCTCTC. Following PCR amplification of cDNA with these primers, the resultant templates were used to generate dsRNA by *in vitro* transcription reaction. After column (Ambion) purification, 15 μg of dsRNA was digested with 15 units of Rnase III at 37°C for 1 h. Digestion products were then purified with a siRNA Purification Unit (Ambion) and siRNA quantified spectrophotometrically. Transfections were carried out using 1.5 μl siPORT Lipid (Ambion) and 25 nM p53 siRNA or Silencer negative siRNA control (Ambion) in 24 well cell culture plates. After 48 h, cells were treated with DON and analyzed for caspase-3 activity.

Statistics. Data were analyzed using SigmaStat for Windows (Jandel Scientific, San Rafael, CA). Data were subjected to one-way ANOVA and pairwise comparisons made by Bonferroni or Student-Newman-Keuls methods. Differences were considered significant at $p < 0.05$.

RESULTS

DON-Induced MAPK Activation Mediates Apoptotic and Survival Pathways in RAW 264.7 Cells

Incubation of RAW 264.7 murine macrophages with 250 ng/ml DON was verified to induce phosphorylation of p38 within 15 min and phosphorylation of these MAPKs were maintained for at least 180 min (Fig. 1). A similar pattern was observed for ERK 1/2 with phosphorylation being most apparent after 30 min. DON at 250 ng/ml induced marked DNA fragmentation in the cells after 6 h. Inclusion of the p38 inhibitor SB203580 blocked DON-induced fragmentation whereas the ERK inhibitor PD98059 enhanced the fragmentation (Fig. 2A). DON-induced DNA fragmentation was dose-dependently inhibited by caspase 3 inhibitor 1 suggesting apoptosis was caspase-3-dependent (Fig. 2B). DON upregulated caspase-3 activation, which was suppressed by the p38 inhibitor (Fig. 3A) but potentiated by the ERK inhibitor (Fig. 3B). The results suggested that DON induced caspase-dependent apoptosis and competing survival pathways via p38 and ERK 1/2, respectively in the macrophage.

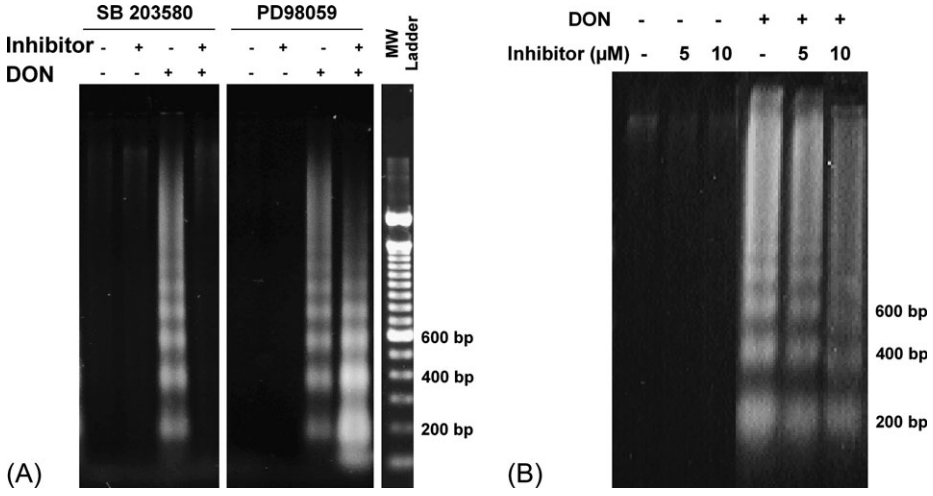

FIG. 2. p38 and caspase-3 mediate and ERK suppresses DON-induced apoptosis. RAW 264.7 cells (5×10^5/ml) were (A) pretreated with or without 5 μM of the p38 inhibitor SB 203580 or 30 μM of ERK inhibitor PD98059 for 30 min before exposure to 250 ng/ml of DON for 6 h or were (B) treated with or without caspase-3 inhibitor I for 30 min and then incubated with or without DON (250 ng/ml) for 3 h. Internucleosomal DNA fragmentation was assessed by agarose gel electrophoresis. Results are representative of three separate experiments.

DON-Induced Apoptosis Is Mediated by the Intrinsic Pathway

The intrinsic pathway of apoptosis involves translocation of BAX to the mitochondrial membrane resulting in depolarization and subsequent cytochrome C release (Marsden and Strasser, 2003). The RAW 264.7 cells treated with DON contained markedly less BAX in cytoplasm then control cells, whereas there was a trend toward increased BAX in the mitochondrial fraction of DON treated cells (Fig. 4A) suggesting the toxin induced translocation of the factor to mitochondria. Consistent with these findings, DON was observed to decrease mitochondrial cytochrome C concentrations but increase the factor in cytoplasm (Fig. 4B). The potential linkage of cytochrome C release to mitochondrial membrane depolarization was assessed with JC-1 dye and flow cytometry. The positive control CCCP caused marked an increase and decrease in JC-1 green and red fluorescence, respectively (Supplementary Data). However, incubation with 500 or 2500 ng/ml of DON for 4 h had no effect. Incubation for shorter (1 h) or longer (6 or 12 h) yielded similar results (data not shown). The results suggest that DON induced apoptosis via the intrinsic mitochondrial pathway but this did not involve mitochondrial depolarization.

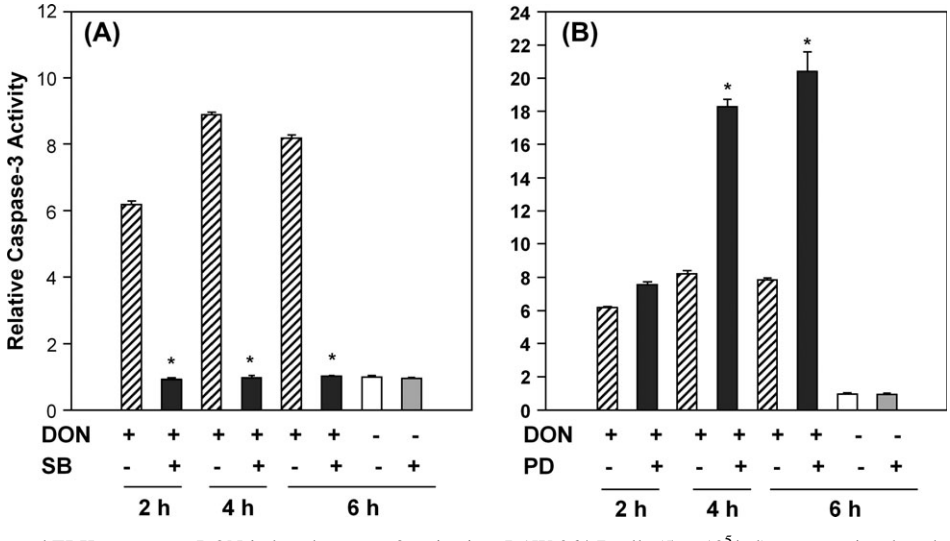

FIG. 3. p38 mediates and ERK suppresses DON-induced caspase-3 activation. RAW 264.7 cells (5×10^5/ml) were pre-incubated with or without (A) 5 μM SB203580 or (B) 30 μM PD 98059 for 30 min followed by the treatment with or without DON (250 ng/ml) for various time intervals. Cell lysates were analyzed with fluorogenic caspase-3 assay. Data are mean ± SEM ($n = 3$). Asterisk indicates significantly different from corresponding cells treated with DON only ($p < 0.05$). Results are representative of three separate experiments.

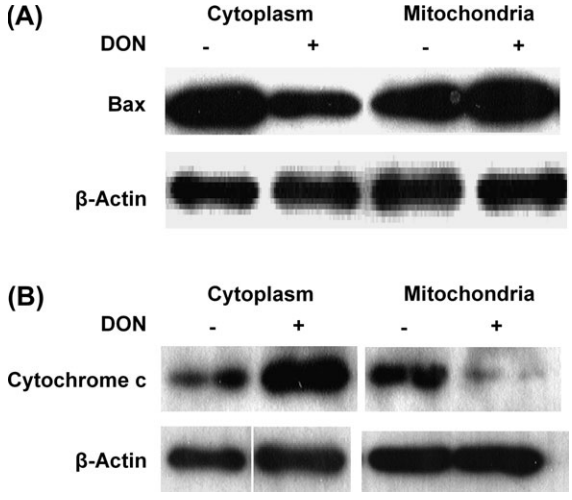

FIG. 4. DON induces Bax translocation to mitochondria and cytochrome C release. RAW cells (5×10^5/ml) were incubated with and without DON (250 ng/ml) for 6 h. Cytoplasmic and mitochondrial fractions were subjected to Western analysis using (A) Bax-specific and (B) cytochrome c-specific antibodies. Membranes were stripped and reprobed with β-actin antibody to verify protein loading. Results are representative of three separate experiments.

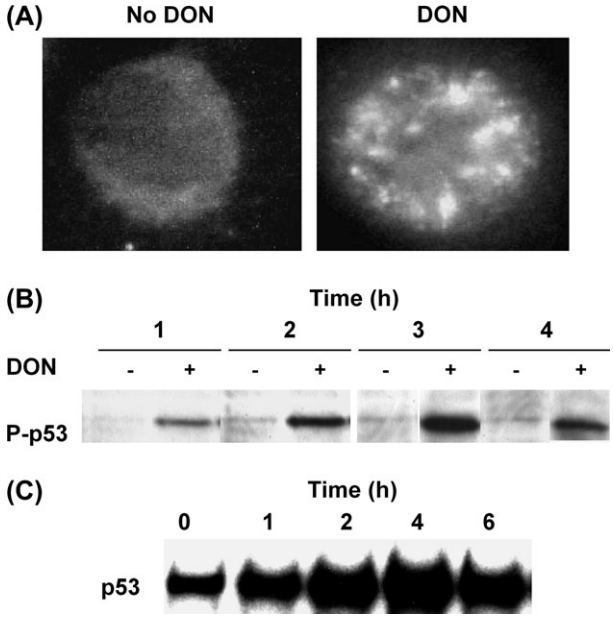

FIG. 5. DON induces p53 phosphorylation and binding activity (A) RAW 264.7 cells grown on minislides and incubated with or without DON (250 ng/ml) for 30 min. Cells were fixed and stained with anti-phospho-p53 (Ser15) followed by goat anti rabbit IgG-FITC. RAW cells (5×10^5/ml) were treated with 250 ng/ml of DON for various time intervals and (B) cell lysates (40 μg) were resolved on SDS-PAGE and subjected by Western blot analysis with antibody specific for phosphorylated p53 (Ser15) or (C) nuclear extracts (10 μg) analyzed by EMSA using p^{32}-labled p53 consensus probe. Results are representative of three separate experiments.

DON Induces p53 Activation

p53 is a critical mediator of apoptosis in stressed cells (Hofseth *et al.*, 2004; Pluquet and Harnaut, 2001) and might similarly contribute to DON-induced cell death. Fluorescence microscopy revealed that exposure of RAW 264.7 cells resulted in phosphorylation of p53 (Fig. 5A). These effects were confirmed by Western analysis of cell lysates with p53 phosphorylation being observed at 1 to 4 h and peaking at 3 h (Fig. 5B). Consistent with these findings was an increase in the capacity of nuclear extracts to bind p53 consensus sequence starting at 1 h and peaking at 3 h (Fig. 5C). The capacity of p53 to catalyze autophosphorylation and activation was confirmed using PFTα, a p53-specific inhibitor (Fig. 6).

DON-Induced Apoptosis Is p53-Dependent

When PFTα was used to assess the role of p53 in DON-induced apoptosis, the inhibitor dose dependently abrogated both caspase-3 activation (Fig. 7A) and DNA fragmentation (Fig. 7B). These findings were further confirmed using p53 siRNA transfection. Sip53 transfection reduced DON-induced caspase-3 activation by approximately 65 percent (Fig. 8A). Similarly siRNA abrogated DON-induced DNA fragmentation (Fig. 8B).

A linkage between p38 and p53 activation was demonstrated using SB203580, which blocked phosphorylation of p21, a substrate of p53 (Fig. 9A) as well as p53 binding to its consensus sequence (Fig. 9B). Thus, in the macrophage, exposure to DON sequentially induced p38 phosphorylation, p53 activation and finally apoptosis via the intrinsic mitochondrial pathway.

DON Induces Two Survival Pathways

Based on the apparent protective effects of ERK 1/2, potential downstream survival pathways were investigated. p90RSK is phosphorylated by ERK 1/2 and capable of phosphorylation and inactivating BAD, a critical factor in the intrinsic apoptotic pathway (Franklin and McCubrey, 2000). DON was found to induce p90RSK (ser 380) phosphorylation within 15 min in RAW 264.7 cells which was maintained for at least 180 min (Fig. 10A). The downstream substrate BAD was also phosphorylated at ser136. The dependency of these pathways on ERK 1/2 was verified by inhibition with PD98059 (Fig. 10B).

In addition to the p90RSK, the kinase AKT (PKB) is known to mediate survival in stressed cells by multiple pathways including BAD phosphorylation (Downward, 2004). DON upregulated AKT phosphorylation at ser 473 within 15 to 30 min after exposure to the toxin (Fig. 11). In addition, phosphorylation of two substrates of AKT, GSK-3β and FKRH were upregulated. Thus, in addition to apoptosis, both p90 RSK and AKT competing survival pathways are induced by DON.

DISCUSSION

DON and other trichothecenes paradoxically can stimulate or suppress immune function by upregulating gene expression

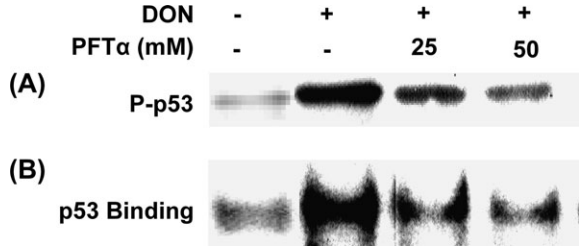

FIG. 6. PFTα, a p53 inhibitor, impairs DON-induced p53 phosphorylation and binding activity. RAW 264.7 cells were incubated with or without PFTα (25 μM or 50 μM) for 30 min and then exposed to DON (250 ng/ml) for an additional 30 min. Cell lysate (40 μg) were analyzed by Western analysis using (A) anti-phospho-p53 (Ser15) antibody for detection of phosphorylated p53. (B) Nuclear extracts (10 μg) were analyzed by EMSA for detection of p53 binding activity. Results are representative of three separate experiments.

or apoptosis, respectively. Pivotal to these opposing effects is the capacity to activate both apoptotic and survival pathways (Fig. 12). The DON concentrations described here (250–500 ng/ml) partially inhibit protein synthesis and have been previously shown to both induce proinflammatory gene expression as well as apoptosis in the macrophage (Zhou *et al.*, 2003a, 2005). Several novel findings are reported herein. First, DON induced apoptosis via a p38-dependent intrinsic mitochondrial pathway. Second, p53 served as the linkage between p38 and the intrinsic apoptotic pathway. Third, both ERK p90RSK and AKT-mediated survival pathways were activated by DON concurrently with apoptosis. The type of immunotoxic response initiated by DON will thus likely depend on whether the balance is tipped toward these apoptotic or survival pathways.

Even though myeloid cells are critical to both innate and acquired immune function, relatively little is known about regulation of their survival and death compared to B and T cells (Marsden and Strasser, 2003). The capacity of DON to prevent or cause macrophage cell death can impact host defense, inflammation, phagocytosis and antigen uptake for presentation and activation of T cells. Indeed, macrophage apoptosis might explain why animals exposed to high doses of trichothecenes exhibit diminished resistance to pathogens such as *Listeria*, *Salmonella*, and *Cryptococcus* as well as diminished phagocytic capabilities (Pestka, 2003). Both intrinsic mitochondrial and extrinsic death receptor pathways are operational in macrophages (Marsden and Strasser, 2003). Our finding that the intrinsic pathway was induced by DON in macrophages is consistent with reported induction of cytochrome C release, as well as caspase-3, -8, and -9 activation by trichothecenes T-2 toxin (Holme *et al.*, 2003; Nagase *et al.*, 2001), fusarenone-X (Miura *et al.*, 2002), satratoxin G (Nagase *et al.*, 2002), and AETD (Pae *et al.*, 2003) in HL-60 cells. These data do not, however, preclude the possibility that TNF-α or other mediators induced by DON could also contribute to the extrinsic apoptotic pathway which would function in parallel to mitochondrial-driven apoptosis.

Extrusion of cytochrome C occurs following disruption of the association of the hemoprotein with cardiolipin which anchors it to the outer surface of the inner mitochondrial membrane (Orrenius, 2004). This can occur after (1) permeabilization of the outer mitochondrial membrane mediated by BAX or other proapoptotic Bcl 2 family proteins, or (2) Ca^{2+}-triggered mitochondrial permeability transition. Although it has been suggested that these two mechanisms are linked, De Marchi *et al.* (2004) recently reported that BAX has no major role in regulating Ca^{2+}-induced mitochondrial permeability transition. This is consistent with our observations that DON mediated apoptosis by the intrinsic mitochondrial pathway without altering mitochondrial membrane potential. However, these results differ from the recent observation that the ribosomal inactivating protein abrin induces mitochondrial membrane depolarization in Jurkat T cells (Narayanan *et al.*, 2004).

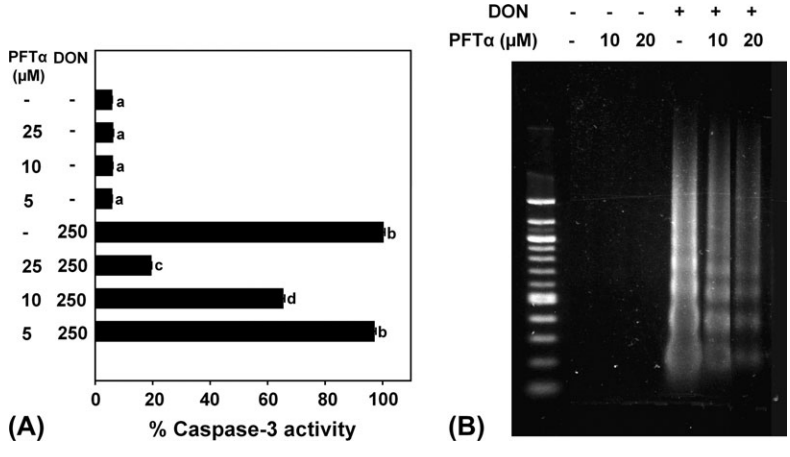

FIG. 7. PFTα dose-dependently reduces DON-induced caspase-3 activity and apoptosis. RAW 264.7 cells (5×10^5/ml) were pretreated with or without PFTα (25 μM) for 30 min and then incubated with DON (250 ng/ml) for 3 h and analyzed for (A) caspase-3 activity by fluoresence assay or (B) DNA fragmentation by agarose gel electrophoresis. Left lane is molecular weight ladder containing 100 bp increments. Results are representative of two separate experiments.

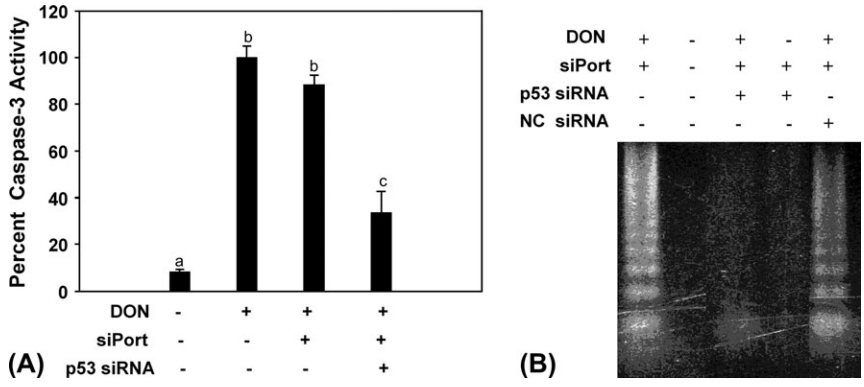

FIG. 8. p53 siRNA suppresses DON-induced caspase 3 activity and apoptosis. RAW 264.7 cells (5×10^5/ml) were transfected with 25 nM of p53 or silencer negative control (nc) siRNA using siPORT lipid. After 48 h, cells were incubated with DON (250 ng/ml) for 3 h and then analyzed for (A) caspase 3 activity assay and (B) DNA fragmentation by agarose electrophoresis. Results are representative of two separate experiments.

p38 and ERK 1/2 activation were essential for inducing the competing apoptotic and survival pathways observed here. Trichothecenes and other translational inhibitors can activate MAPKs by an incompletely understood mechanism known as the ''ribotoxic stress response'' (Iordanov *et al.*, 1997; Laskin *et al.*, 2002). Recently, we have observed that both double-stranded RNA activated protein kinase (PKR) (Zhou and Pestka, 2003a) and Src-family tyrosine kinases (Zhou and Pestka, 2005) are essential upstream signals for MAPK activation by DON and other ribotoxins. Both have the potential to be physically linked to the ribosome and endoplasmic reticulum and thus might be capable of transducing a signal from the ribosome to a downstream MAPK. Impairment of PKR (Zhou *et al.*, 2003a) or the Src Hck (Zhou *et al.*, 2005) with inhibitors or interference with gene expression in macrophages have been shown previously to inhibit DON-induced apoptosis.

p53 is a central regulator of cell death which acts pleiotropically by transcriptional-dependent and -independent mechanisms (Oren, 2003). p53 can upregulate transcription of apoptosis-dependent genes related to the extrinsic pathway such as Fas, DR5, and PERP as well as the intrinsic pathway including the BH3-only proteins Bax, Noxa, Puma, and Bid (Haupt *et al.*, 2003). p53 can also transactivate genes associated with apoptosome activation (Apaf-1) and caspase 6. In contrast, Chipuk *et al.* (2004) reported that a transcription-independent mechanism whereby p53 functions analogously to the BH3-only proapoptotic Bcl-2 proteins in the activation of Bax and triggering of apoptosis. Erster *et al.* (2004) found in sensitive organs *in vivo* that mitochondrial p53 accumulation occurs soon after a death stimulus triggering a rapid first wave of apoptosis that is transcription-independent and precedes a second slower wave of transcription-dependent apoptosis. It is likely that p53-independent mechanisms for DON-induced apoptosis are also possible based on our previous findings that the toxin can induce apoptosis in U937

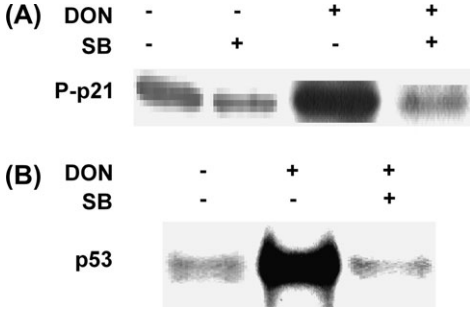

FIG. 9. p38 inhibitor SB 205580 inhibits DON-induced p21 phosphorylation and p53 binding activity. RAW cells (5×10^5/ml) incubated with SB 203580 (5 μM) for 30 min and then with DON (250 ng/ml) for 30 min. (A) Cell lysates were subjected to Western blot using an anti-phospho-p21 antibody. (B) Nuclear extracts were subjected to EMSA analysis for detecting p53 binding activity. Results are representative of three separate experiments.

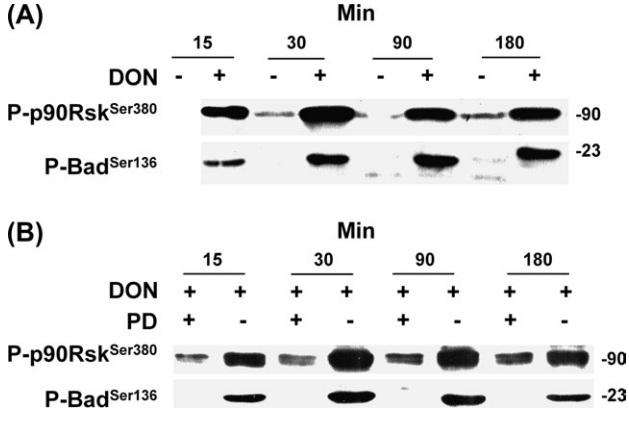

FIG. 10. ERK inhibitor PD 98059 impairs DON-induced p90Rsk and Bad phosphorylation. RAW 264.7 cells (5×10^5/ml) were (A) treated with or without DON (250 ng/ml) for various time intervals or (B) treated with DON after pretreatment with or without 30 μM PD 98059 for 30 min and then with DON. Cell lysates were resolved on SDS-PAGE and subjected to Western blot analysis with antibody specific for phosphorylated p90Rsk (Ser380) or phosphorylated Bad (Ser136). Numbers on right refer to MW in KD. Results are representative of two separate experiments.

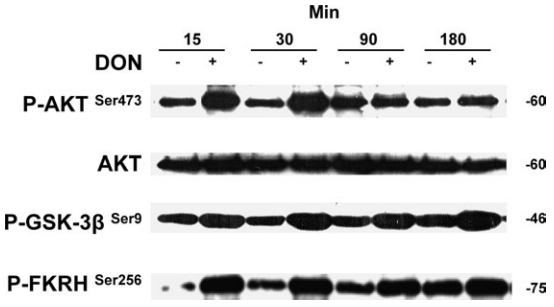

FIG. 11. DON induces the phosphorylation of AKT Ser473 and AKT targets FKRH and GSK-3β. RAW 264.7 cells (5×10^5/ml) were treated with DON (250 ng/ml) for various time intervals. Cell lysates were resolved on SDS-PAGE subjected to Western blot analysis with antibodies specific for phosphorylated FKRH (Ser256) or phosphorylated GSK-3β (Ser9). Numbers on right refer to MW in KD. Results are representative of two separate experiments.

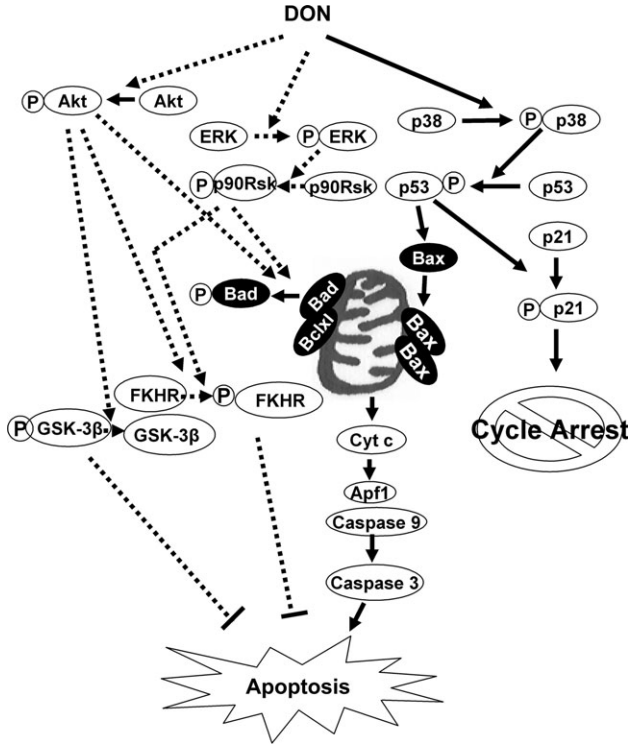

FIG. 12. Summary of putative competing apoptotic and survival signaling pathways in the macrophage induced by DON. DON-induced p38 activation drives p53 activation with resultant p21 phosphorylation leading to cycle arrest and translocation of BAX to mitochondria. Release of cytochrome C from mitochondria initiates caspase-3 mediated apoptosis. Two concurrent survival pathways mediated by ERK 1/2 driven phosphorylation of p90RSK and by AKT are induced by DON. The extent of apoptosis induction by DON will be determined by the balance of these competing pathways.

monocytes which lack functional p53 (Zhou and Pestka, 2003a). Further investigation is needed on whether DON-induced p53 activation induces transcriptional-dependent or -independent apoptosis. It was interesting to note that p53 activation also mediated p21 phosphorylation. This action can cause cell cycle arrest which is widely described effect of trichothecenes (Fornelli *et al.*, 2004).

Several mechanisms downstream of ERK 1/2 have been reported to prevent apoptosis (Franklin and McCubrey, 2000). Notably p90Rsk, a substrate of ERK 1/2, is affected by DON. p90Rsk is known to phosphorylate Bad which can decrease interaction of this protein with anti-apoptotic Bcl2 family members thereby inhibiting mitochondrial release of cytochrome C. p90Rsk can also mediate CREB phosphorylation which promotes cell survival via transactivation of anti-apoptotic genes such as Bcl-XL (Bonni *et al.*, 1999). In addition to these downstream mechanisms, MEK, the kinase upstream of ERK 1/2 and the actual target of PD98059, also phosphorylates Bad thereby releasing anti-apoptotic Bcl proteins.

The data presented here demonstrated that DON induced phosphorylation and activation of AKT as well as phosphorylation of its substrates GSK-3β and FKRH. The AKT anti-apoptotic pathway mediated is perhaps the most widely studied survival pathway and has many facets (Downward, 2004; Franklin and McCubrey, 2000). Like p90Rsk, AKT phosphorylates Bad and blocks cytochrome C release from mitochondria (del Peso *et al.*, 1997; Kennedy *et al.*, 1999). AKT also promotes phosphorylation of the forkhead transcription factor, FKRH, which results in cytoplasmic retention of FKRH suppressing transcription of pro-apoptotic genes (Brunet *et al.*, 1999; Downward, 2004). GSK3 (glycogen synthease kinase-3) is inhibited following phosphorylation by AKT thus promoting storage of glucose as glycogen (Cross *et al.*, 1995) which promotes survival through a poorly understood mechanism (Downward, 2004). Another proposed anti-apoptotic mechanism for AKT is activation of the E3 ubiquitiin ligase MdM2 which inhibits both level and function of p53 (Ashcroft *et al.*, 2002; Gottlieb *et al.*, 2002; Mayo and Donner, 2001). The mechanism by which DON activates AKT is unknown but might involve PI3K (Downward, 2004). One possibility is that the N-terminal cleavage product of RasGAP generated by executioner caspases can directly activate the Ras-PI3K–AKT survival pathway (Yang *et al.*, 2004). Clearly, further exploration of the linkage between PI3K and ribotoxic stress response is needed.

In conclusion, DON induced competing apoptotic and survival pathways in RAW 264.7 macrophages (Fig. 12). There have been over 1500 research publications employing this cell line as a mimic of macrophages during the past 25 years suggesting the veracity of this model. Nevertheless, it will be desirable in the future to validate whether these aforementioned pathways similarly occur in primary macrophage cultures. Towards this end, we have indeed observed that DON induces p38, ERK, and AKT phosphorylation in peritoneal macrophages (data not shown). Future work should also focus on the capacity of other ribotoxins such as abrin to also mediate apoptotic and survival pathways in the macrophage.

SUPPLEMENTARY DATA

Supplementary data are available online at www.toxsci.oupjournals.org.

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